

Role of Specialized Microvilli and the Fertilization Envelope in the Spatial Positioning of Blastomeres in Early Development of Embryos of the Starfish *Astropecten scoparius*

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Abstract. In the eggs of a wide range of animal species, various factors that determine the blastomeres' presumptive fate are known to locate unevenly within the egg. In the embryos of these animals, cleavage occurs not just to increase cell numbers, but also to distribute the factors to the respective blastomeres, resulting in cell specialization at the later stages. In the early cleavage stages, before the establishment of a device such as desmosomes to directly join the blastomeres, some other means is needed to keep the blastomeres together and maintain the relative positions among them. In this study, we found that the embryos of the starfish *Astropecten scoparius* lack the hyaline layer seen in sea urchin embryos and that blastomeres adhere to the fertilization envelope (FE) *via* filamentous cellular projections (fixing processes). Electron microscopy revealed the fixing processes to be specialized microvilli formed, after the elevation of the FE, by the elongation of short microvilli that pre-exist in unfertilized eggs. After the first cleavage, the two blastomeres separate from each other and finally attach to the FE. In the subsequent cleavages, the blastomeres undergo repeated cell division without separating from the FE. Between the blastomeres and the FE, only shortened fixing processes were observed. Destruction of the fixing processes caused release of the blastomeres from the FE and disturbance of the relative positions of the blastomeres, resulting in abnormal development of the embryos. These observations suggest that the fixing process is a device to keep the egg placed centrally in the FE up to the

first cleavage, and after the first cleavage and beyond to anchor the blastomeres to the FE so that the FE can be used as a scaffold for morphogenesis. Electron microscopy also suggests that the inner layer of the FE, which is derived from the contents of cortical granules, reinforces the adhesion of the fixing processes to the FE. Immuno-electron microscopy, using an antibody against sea urchin hyaline layer, showed that the inner layer of the FE of starfish eggs and the hyaline layer of sea urchin eggs, which are both derived from cortical granules, contain some common elements.

Introduction

In mammals, "compaction" of the embryo occurs at the 8-cell stage, leading to binding of the blastomeres together (Tarkowski and Wroblewska, 1967; Hillman *et al.*, 1972; Fleming *et al.*, 1989). The presumptive fate of the blastomeres is first determined at this stage. In these embryos there is accordingly a close relationship between the formation of a binding device for the blastomeres and their differentiation.

In most invertebrate embryos, however, the presumptive fate of each blastomere is determined at a much earlier stage than the formation of intercellular binding devices such as desmosomes (*cf.* Kuraishi and Osanai, 1989; Kiyomoto and Shirai, 1993a, 1993b). It is known that a number of morphogenetic determinants, which play a role in the determination of the blastomeres' presumptive fate, are located unevenly within the egg of these animals (Boveri, 1910; Hörstadius, 1935; Anderson and Nusslein-Volhard, 1984). In those embryos, cell division occurs not just to increase cell numbers, but also to distribute the various determinants

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to the respective blastomeres and induce cell specialization. If the distribution of the determinants is upset, or some of the blastomeres are removed, and the spatial positions of blastomeres are changed, normal development of the embryo is disturbed (Chabry, 1887; Hörstadius, 1935; Gilbert, 1994). Animal development provides examples of many ways to maintain the relative positions among blastomeres in the period before the formation of intercellular binding devices. For example, in the embryos of jellyfish (Dan and Dan, 1947) and hydra (Martin *et al.*, 1997), species that lack an investing layer, spinning processes or ladderlike membrane structures are formed, respectively, between blastomeres. In sea urchins, a hyaline layer is formed on the surface of the egg immediately after fertilization (Endo, 1961), and this layer holds the outer surface of the embryo in place by means of microvilli (Dan and Ono, 1952; Endo, 1961). If the embryo is exposed to antibodies against the hyaline layer, normal morphogenesis is inhibited (Adelson and Humphreys, 1988). In sea urchin embryos, therefore, the relative positions among the blastomeres are maintained by the hyaline layer and microvilli (Burgess and Schroeder, 1977; Adelson and Humphreys, 1988). In the embryos of starfish, which belong to the same class of Echinodermata as sea urchins, a hyaline layer is not formed in the early cleavage stages (Matsunaga *et al.*, 2000), so some other means must exist to unite the blastomeres. In the embryos of the starfish *Astropecten scoparius*, we observed the individual blastomeres lining up along the inner surface of the fertilization envelope and found threadlike structures (fixing processes) connecting the blastomeres to the fertilization envelope. Normal morphogenesis was prevented if the connection between the blastomeres and the fertilization envelope was severed, suggesting that the fertilization envelope and the fixing process play an important role in maintaining the relative positions of the blastomeres in *A. scoparius* embryos. In this study, we examined the shape, character, and function of these fixing processes.

Materials and Methods

Handling of gametes

The starfish *Astropecten scoparius* was collected from Tateyama Bay in Chiba Prefecture, Japan. Isolated ovaries were treated with calcium-free seawater for 20–30 min, and then ovulation was induced by returning them to filtered normal seawater (NSW), yielding immature oocytes without the follicular envelope (Nemoto *et al.*, 1980). “Dry sperm” taken from isolated testes were diluted with NSW just before use. Maturation division of oocytes was induced with 2 μ M of 1-methyladenine (1-MeAde; Sigma-Aldrich, St. Louis, MO) dissolved in NSW (Kanatani, 1969). At 20 °C, germinal vesicle breakdown (GVBD) and extrusion of the first and second polar bodies occurred, respectively, at about 25, 60, and 90 min after treatment with 1-MeAde. The maturing oocytes were fertilized 40 min after treatment with

1-MeAde. The fertilized oocytes and embryos were rinsed once with NSW and then cultured at 20 °C. The average diameters of the oocytes and the fertilization envelope were 210 and 300 μ m, respectively.

Light microscopy

Oocytes, eggs, and embryos were flattened to a 180- μ m thickness and examined under a differential interference-contrast microscope (Axiophoto; Zeiss, Oberkochen, Germany). Microphotographs were taken with Neopan 400 Presto film (Fuji Photo Film, Tokyo, Japan).

Transmission electron microscopy

Two fixing methods were used in this study. A quick-freeze and freeze-substitution method was mainly used for examination of cytoskeletons, and chemical fixation was used for examination of other structures.

Chemical fixation. Oocytes and embryos were first fixed in 2% glutaraldehyde (TAAB, Berkshire, England) dissolved in NSW for 2 h at room temperature, then in Karnovsky's fixative (Karnovsky, 1965) for 2 h at room temperature, followed by 10 h at 4 °C. After being rinsed several times with 0.1 M sodium-cacodylate buffer (pH 7.2), they were then fixed in 1% osmium tetroxide (Polyscience, Warrington, PA) dissolved in 0.1 M sodium cacodylate for 60 min on ice (Steinbeck *et al.*, 1993). The fixed specimens were rinsed twice with distilled water (DW) and then dehydrated with ethanol.

Quick-freeze and freeze-substitution fixation. Oocytes were first treated with 15% butanediol (Wako Pure Chemical, Tokyo, Japan) diluted with NSW for 20 min on ice (Reimer and Crawford, 1997), and then they were collected with a hand-driven centrifuge. A 20- μ l aliquot of the specimen was placed on an electron microscopy grid. Excess NSW was drawn off with a piece of filter paper, and the specimens were frozen by immersion for 10 s in liquid propane that had itself been cooled with liquid nitrogen. The frozen materials were placed in 4% osmium tetroxide/acetone cooled to -90 °C. Five days later, the specimens were first brought up to -20 °C and then to 4 °C for 2 h each. They were finally placed at room temperature (Burke *et al.*, 1998) and dehydrated using acetone.

Embedding and examination. The dehydrated specimens were embedded in epoxy resin (Kushida, 1980), and ultrathin sections (90 nm in thickness) were produced using an ultramicrotome (MT-XL, RMC Inc., Tucson, AZ). The sections were stained with uranyl nitrate and lead acetate for 2 min each and examined under a JEOL 1010 transmission electron microscope (JEOL, Tokyo, Japan).

Immuno-electron microscopy

Ultrathin sections made from the chemically fixed specimens underwent 5 min of etching with 3% sodium meta-

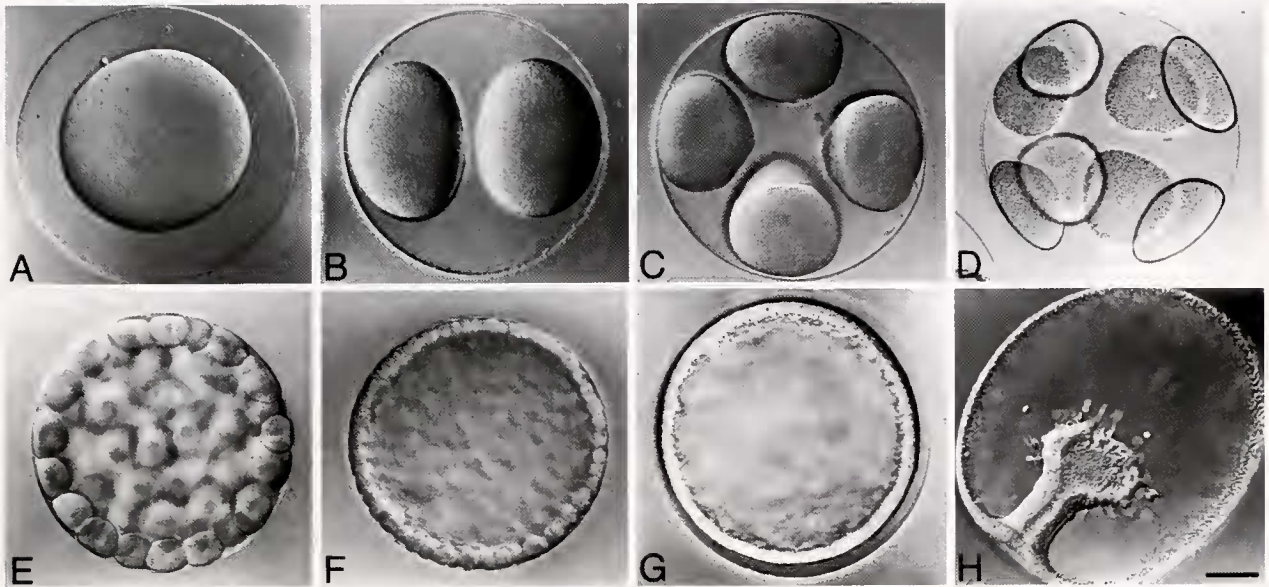


Figure 1. Development of *Astropecten scoparius* embryos derived from oocytes fertilized 40 min after treatment with 1-MeAde. (A) Just after the completion of meiotic division (2 h 10 min after 1-MeAde treatment). A second polar body is seen at the upper left of the egg. (B) The 2-cell stage just before the second cleavage (2 h 40 min). The two blastomeres moved toward and adhered to the fertilization envelope, producing space between them. (C) The 4-cell stage just before third cleavage (3 h 10 min). The blastomeres are separated from each other along the fertilization envelope in a direction perpendicular to the cleavage plane. (D) The 8-cell stage just after cleavage (3 h 40 min). The blastomeres adhere to the fertilization envelope, and adjacent blastomeres have become widely separated. (E) The 2^6 -cell stage (6 h 10 min). The blastomeres continued to undergo cell division without separating from the fertilization envelope, and eventually lined up along the inside surface of the fertilization envelope. (F) The 2^8 -cell stage (7 h 10 min). A blastula consisting of a single cell layer. (G) A rotating blastula just before hatching (12 h 40 min). A space is seen between the fertilization envelope and the embryo. (H) A gastrula (24 h 40 min). Scale bar: 50 μ m.

periodate. After being rinsed with DW, they were treated with an antibody raised against sea urchin hyaline layer (Yazaki, 1968) diluted in 0.2% Tween 80 for 5 min at room temperature. After another rinse with DW, the sections were treated with an anti-rabbit IgG antibody (Sigma-Aldrich) bound to protein A-colloidal gold (20 nm in diameter), diluted 1/40 in 0.2% Tween 80, for 5 min (Morris and Ciruolo, 1997). These sections were not stained with lead acetate or uranyl nitrate.

Results

Cleavage and development of fertilized eggs

Oocytes fertilized immediately following germinal vesicle breakdown (GVBD) extruded a second polar body 1½ h after treatment with 1-MeAde, completing their maturation division (Fig. 1A). As with the embryos of other starfish species, elevation of the fertilization envelope continued for about 2 h after fertilization. The first cleavage occurred some 40 min after the formation of the second polar body. After the first cleavage, the two blastomeres moved in a direction perpendicular to the cleavage plane, as if to separate from each other. Some 15–20 min after the first cleavage, the two blastomeres impressed themselves into

the fertilization envelope at the surface of their mitotic poles (Fig. 1B). As the area of contact between the blastomeres and the fertilization envelope became progressively larger, the blastomeres changed their shape to ellipsoids with the long axis in the animal-vegetal pole direction, producing space between them. The second cleavage occurred some 30 min after the first. After this cleavage, as after the first, space was produced between the blastomeres (Fig. 1C). The cleavage plane for the third cleavage was formed at right angles to the first and second cleavage planes. After this division also, the blastomeres did not separate from the fertilization envelope (Fig. 1D). The blastomeres at this stage adhered to the fertilization envelope over a wide area and had become flattened. Adjacent blastomeres had become widely separated from each other. A hyaline layer could not be distinguished. At the 16-cell stage and beyond, blastomeres continued to undergo cell division without separating from the fertilization envelope, and they lined up along its inside surface (Fig. 1E). As a result, the embryo did not go through the cell-mass, "morula" shape, but became a blastula consisting of a single cell layer (Fig. 1F). When hatching commenced, a space developed between the fertilization envelope and the embryo, and the embryo began to spin around (Fig. 1G). Gastrulation began about 3 h

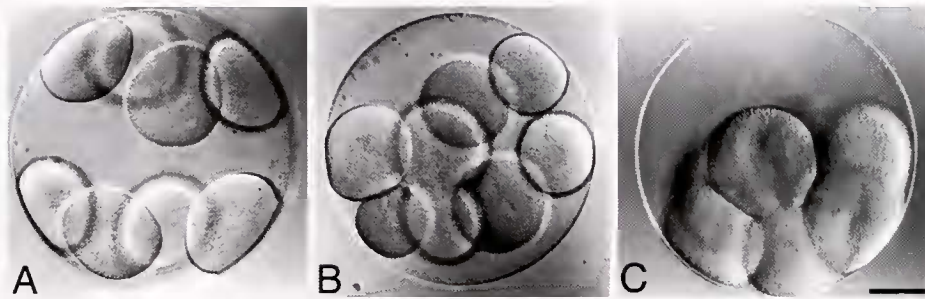


Figure 2. Effect of acidified seawater (pH 4) treatment for 1 min at the 8-cell stage on the alignment of blastomeres. (A) Just before the treatment (3 h after fertilization). Blastomeres show a flattened shape, attaching to the fertilization envelope. (B) One minute after the treatment. Blastomeres changed from ellipsoid to spherical, and the area of the cell surface in contact with the fertilization envelope became smaller. (C) After brief centrifugation. Blastomeres are gathered to the centrifugal end inside the fertilization envelope. Scale bar: 50 μm .

after hatching. The embryos developed to mid-gastrulae 10 h after hatching (Fig. 1H). Development beyond this stage was described by Oguro *et al.* (1976).

Morphogenesis of embryos that have lost attachment between blastomeres and the fertilization envelope

When embryos at the 8-cell stage (3 h postfertilization) were subjected to a 1-min pulse treatment with acidified seawater adjusted to pH 4 with HCl, blastomeres changed from a flattened shape to a globular one, and the area of the cell surface in contact with the fertilization envelope became smaller (compare Fig. 2A with Fig. 2B). Blastomeres also became globular when the fertilization envelope was removed. When embryos treated with acidified seawater were returned to NSW and then gently spun with a hand-driven centrifuge, blastomeres were gathered at the centrifugal end inside the fertilization envelope (Fig. 2C). In untreated embryos, however, the arrangement of the blastomeres was not disturbed even by such centrifugation. These results indicate that the attachments between the fertilization envelope and the blastomeres are severed by treatment with acidified seawater.

Even after embryos pulse-treated with acidified seawater were returned to NSW, the blastomeres did not restore their connection with the fertilization envelope but remained globular, not returning to their normal flattened shape. At 24 h postfertilization, at which time control embryos had formed gastrulae, the embryos treated with acidified seawater alone were morphologically abnormal, many with distorted shapes or abnormal archenterons, and some embryos had multiple archenterons (Fig. 3A, B). The effects of treatment with acidified seawater were especially marked if conducted between 3 and 4.5 h postfertilization (8- to 64-cell stage). No morphological abnormalities were seen in embryos treated with acidified seawater 6.5 h postfertilization (2⁹-cell stage) (Fig. 3C), and normal gastrulae were formed 24 h postfertilization (Fig. 3D). The effect of acidified seawater treatment was no longer observed when it

was done with embryos 6.5 h postfertilization and beyond. These results indicate that in the early stages of cleavage, blastomeres in *A. scoparius* embryos maintain their spatial positions relative to each other by means of the fertilization envelope.

The mechanism of anchoring blastomeres to the fertilization envelope

The above observations suggest the existence of some means that attaches blastomeres to the fertilization envelope. Within a minute postinsemination, when the fertilization envelope could be identified, a number of particles were observed in the perivitelline space (perivitelline-space particles) (Fig. 4A). A few minutes after insemination, many thin processes connecting the fertilization envelope and the egg proper could be seen (Fig. 4B, arrow). These processes will hereafter be referred to as fixing processes. These fixing processes elongated rapidly with elevation of the fertilization envelope and were located over the entire surface of the egg. The perivitelline-space particles moved along the fixing processes towards the fertilization envelope (Fig. 4B, arrowheads), reaching it by several minutes after insemination, by which time very few could be seen in the perivitelline space (Fig. 4C). The perivitelline-space particles adhering to the fertilization envelope eventually disappeared, leaving a smooth inner surface (Fig. 4C). Around this time, the basal part of the process assumed the shape of a cone (Fig. 4C, white arrowheads). After the first cleavage, fixing processes could still be seen between the blastomeres and the fertilization envelope but not on the surface of the cleavage furrow (Fig. 4D; *cf.* Fig. 5A). The length of the fixing processes varied with the distance between the egg surface and the fertilization envelope; the longest, some 60 μm , was seen in the vicinity of the cleavage furrow (Fig. 4D). Near the boundary between the areas of the blastomeres touching the fertilization envelope, fixing processes were short and straight, with no bending being seen. Short

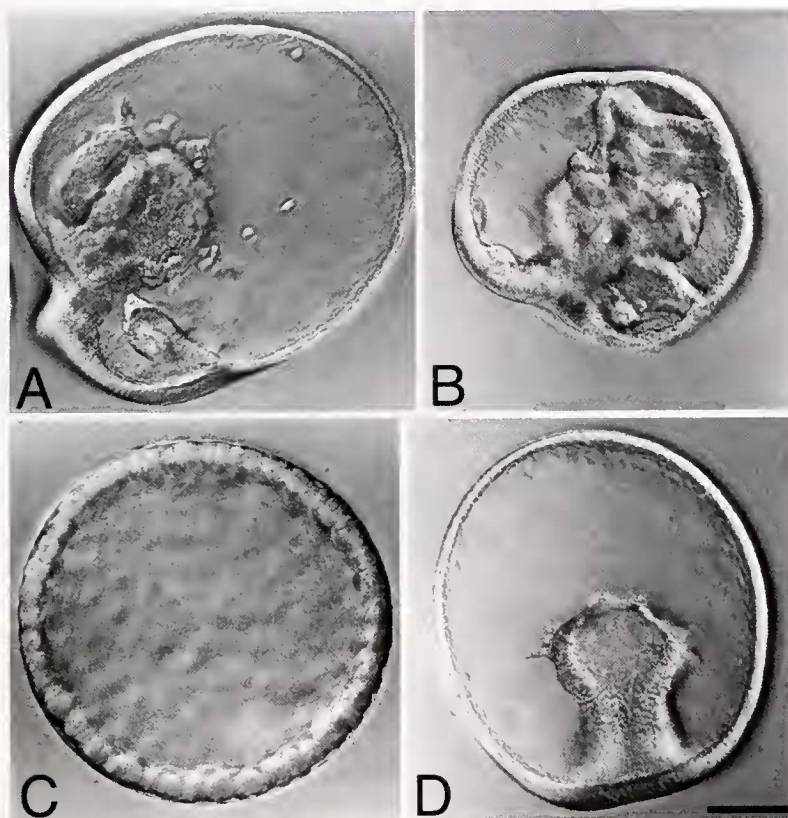


Figure 3. Development of embryos treated with acidified seawater. (A, B) Embryos cultured in normal seawater for 21 h after 1-min pulse treatment with acidified seawater at the 8-cell stage (3 h after fertilization). (A) An embryo with multiple archenterons. (B) An embryo showing abnormal morphology. (C) An embryo just after acidified-seawater treatment at the 2⁹-cell stage (6.5 h after fertilization). No marked changes in morphology are observed (*cf.* Fig. 1F). (D) An embryo from the same batch of embryos as in C, cultured for 17.5 h in normal seawater after the treatment, showing normal morphology of gastrula. Scale bar: 50 μ m.

fixing processes were also observed in embryos at later stages of development (Fig. 5A, white arrowheads).

In embryos at the 8-cell stage, blastomeres still adhered to the fertilization envelope over a wide area and were ellipsoid (Fig. 5A; *cf.* Fig. 2A). Fixing processes (white arrowheads), near the boundary between the areas of the blastomeres touching the fertilization envelope, were thin and straight. After a 1-min pulse treatment with acidified seawater (Fig. 5B), fixing processes were badly damaged and became deformed and thicker (arrowheads) than those of embryos before treatment; they looked as if they had lost their rigidity. Blastomeres separated from the fertilization envelope and became roughly spherical. These observations suggest that the fixing processes are the way that blastomeres are anchored to the fertilization envelope.

Transmission electron microscopic examination of fixing processes

Microvilli, some 700 nm in length, were identified embedded in the vitelline coat on the surface of both immature (Fig. 6A) and maturing unfertilized oocytes (Fig. 6B). At

30 s postfertilization, the tips of the microvilli were connected to the vitelline coat that was transforming into the fertilization envelope (Fig. 6C, arrows). Subsequently, the microvilli elongated with the elevating of the fertilization envelope (Fig. 6D, E). The fixing processes seen under light microscopy (Figs. 4 and 5) are therefore considered to be specialized microvilli. These specialized microvilli sprout from all over the surface of the egg. At early cleavage stages, short fixing processes some 500 nm in length were seen on the surface of the blastomeres adhering to the fertilization envelope (Fig. 6F), and the tips of those processes were connected with the fertilization envelope (arrows). No extracellular matrix surrounding the embryo, such as a hyaline layer, was observed (Fig. 6D–F). Some membranous structures (white asterisk) were sometimes seen in the space between the blastomeres and the fertilization envelope.

In the specimens prepared by the quick-freeze and freeze-substitution method, bundles of fibers could be identified within microvilli in immature oocytes (Fig. 6A). After oocyte maturation, these fiber bundles extended in the direc-

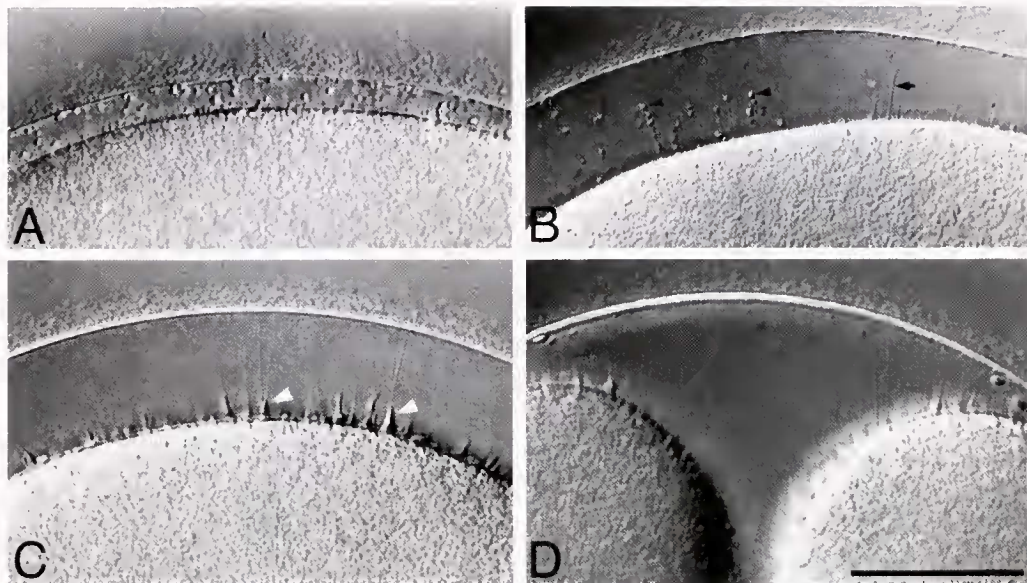


Figure 4. Surface change of eggs immediately after fertilization (inseminated 40 min after 1-MeAde treatment). (A) Forty seconds after insemination. A number of particles are observed in the perivitelline space (perivitelline-space particles). (B) Two minutes after insemination. Note thin processes (fixing processes; arrow) connecting the fertilization envelope and the egg. Perivitelline-space particles (arrowheads) moved along the fixing processes towards the fertilization envelope. (C) Three minutes after insemination. Note the prominent cone-like shape at the bases of the fixing processes (white arrowheads). Very few perivitelline-space particles could be seen in the perivitelline space. (D) The 2-cell stage (3 h after insemination). Fixing processes were elongated in the vicinity of the cleavage furrow and were shortened near the boundary between the areas of the blastomeres touching the fertilization envelope. Scale bar: 50 μm .

tion of the cytoplasm (Fig. 6B, white arrow), reaching a length of 2 μm . The diameter of these fibers was 5–6 nm, so they can be considered microfilaments. Crossing the microfilament bundle, repeated bands were observed (Fig. 6B, black arrowheads).

We could not examine microfilaments in the fixing processes by transmission electron microscopy, because the fixing processes were not preserved by the quick-freeze and freeze-substitution fixation that effectively preserves microfilaments. To get information on these microfilaments, we examined the effects of cytochalasin D on the fixing processes. When the cytochalasin was applied to fertilized eggs, intact fixing processes disappeared within several minutes of treatment, and only fragmented fixing processes were observed in the perivitelline space (Fig. 7A). In embryos pulse-treated with the cytochalasin at early cleavage stages, the fixation of blastomeres on the fertilization envelope were severely damaged, and the blastomeres changed shape from flattened to globular (compare Fig. 7B with Figs. 1D and 2A). These results suggest the presence of microfilaments in the fixing processes.

Immuno-electron microscopic examination of the perivitelline-space particles

In unfertilized maturing oocytes, granular structures with fairly high electron density could be seen along the cortex (Fig. 8A, asterisks). On fertilization, these granules opened

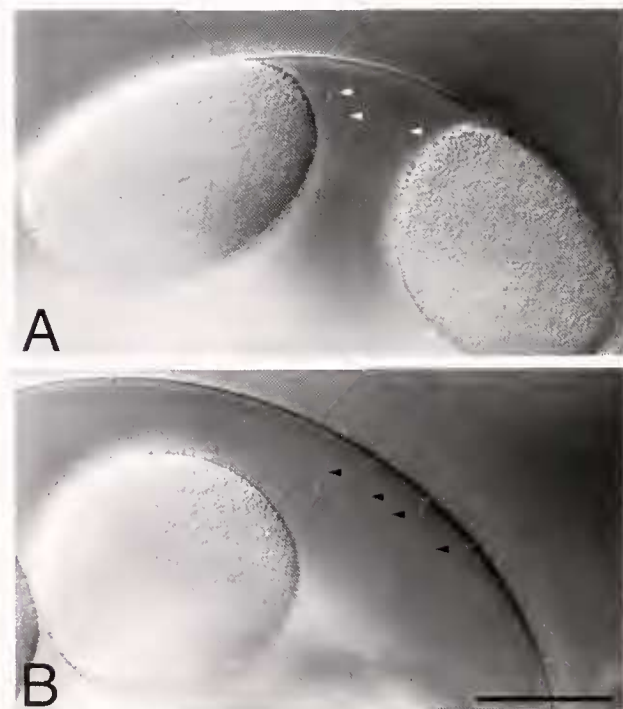


Figure 5. Changes of fixing processes after treatment of 8-cell stage embryos with acidified seawater. (A) An egg before the treatment. Fixing processes are straight and thin (white arrowheads). (B) Two minutes after the treatment. Fixing processes became thicker and were badly damaged (black arrowheads), and the blastomeres changed their shape from ellipsoid to spherical. Scale bar: 50 μm .

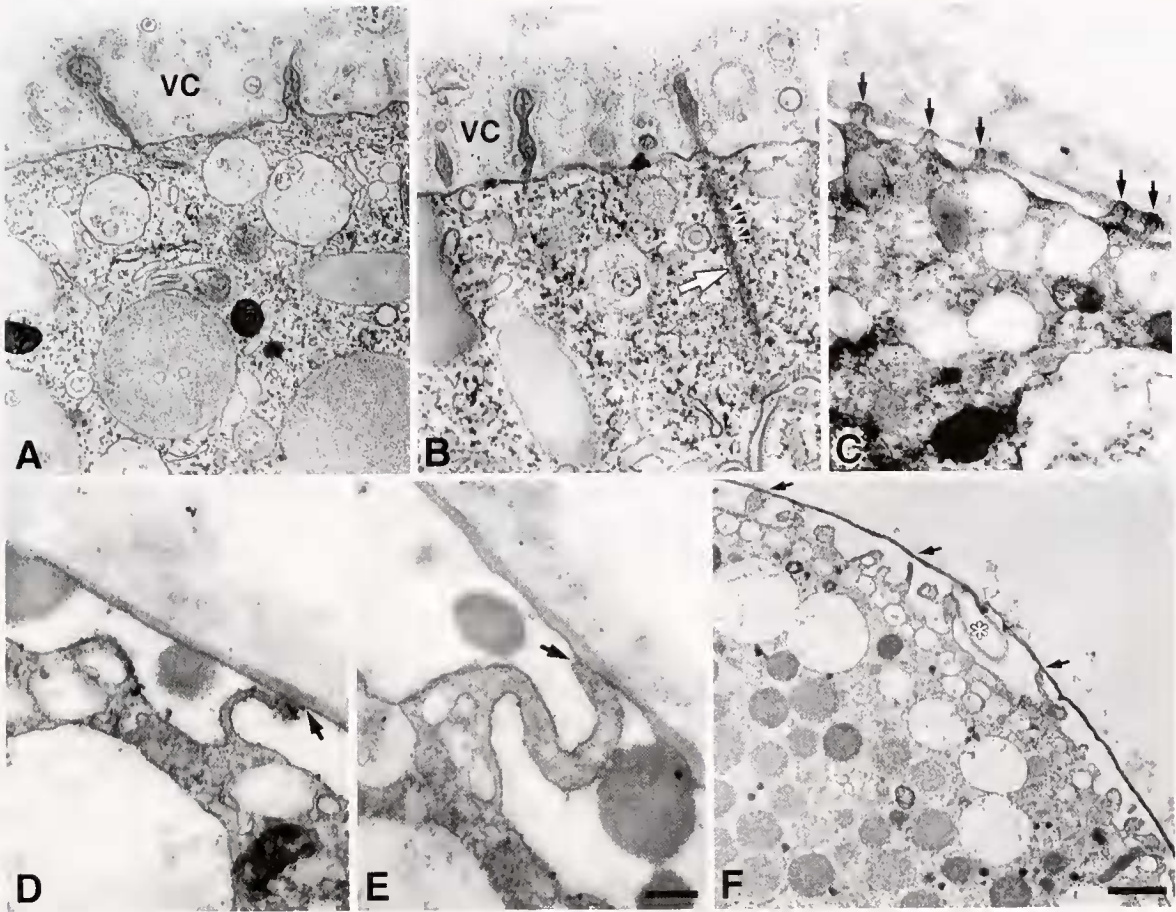


Figure 6. Transmission electron microscopy of microvilli and fixing processes. (A, B) Quick-freeze and freeze-substitution fixation. (C-F) Chemical fixation. (A) An immature oocyte. Microvilli were identified embedded in the vitelline coat (VC) on the surface of the oocyte. Note bundles of fibers within microvilli. (B) A maturing oocyte 40 min after I-MeAde treatment. Fiber bundles inside microvilli extend into the cytoplasm (white arrow). On the fiber bundles, some repeated strips are observed (black arrowheads). (C) Thirty seconds after insemination (40 min after I-MeAde treatment). The tips of microvilli (arrows) were connected to the vitelline coat that was transforming into the fertilization envelope. (D) One minute after insemination. Microvilli were lengthening with the elevation of the fertilization envelope, thereby transforming themselves into the fixing processes. A fixing process attached to the electron-dense innermost layer of the fertilization envelope (arrow). (E) Two minutes after insemination. The length of microvilli increased compared to that in D. A fixing process attached to the electron-dense innermost layer of the fertilization envelope (arrow). (F) The 8-cell stage. Short fixing processes were observed on the blastomere surface adherent to the fertilization envelope. The tips of the processes were connected with the fertilization envelope (arrows). Scale bars: 500 nm (A-E), and 2 μ m (F).

onto the cell surface and discharged the contents into the perivitelline space (Fig. 8B-D; cf. Fig. 6D, E), resulting in their disappearance from the cytoplasm (Fig. 6F). These granules are therefore considered to be cortical granules. Following exocytosis of the cortical granules, electron-dense particles appeared in the perivitelline space, indicating that the particles correspond to the perivitelline-space particles observed under light microscopy (Fig. 4A, B) and are derived from the cortical granules. To confirm this, immuno-electron microscopy was performed using an antibody against the sea urchin egg's hyaline layer (Yazaki, 1968), which is known to derive from the cortical granules (Endo, 1961). As shown in Figure 8A, gold particles are

present inside the cortical granules (asterisks) in unfertilized oocytes. After fertilization, gold particles could be seen specifically in the cortical granule contents that were just being discharged outside the egg (Fig. 8B, asterisk). After their discharge into the perivitelline space (Fig. 8C, asterisk), the cortical granule contents, recognizable by the gold particles, reached the fertilization envelope (Fig. 8D, asterisk). As can be clearly seen in Figures 6 and 8, there is no hyaline layer over the egg surface, and gold particles cannot be identified outside the cell surface (Fig. 8C, D). As the perivitelline-space particles began to arrive at the fertilization envelope, a layer with fairly high electron density appeared on the inner part of the fertilization envelope, to

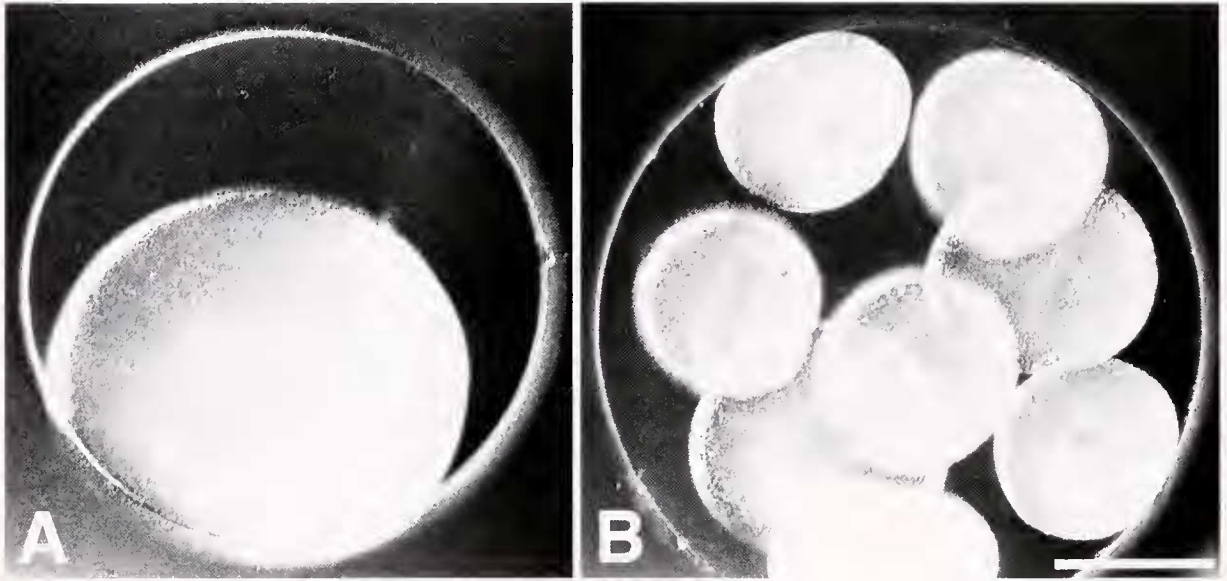


Figure 7. Effects of cytochalasin D on the fixing processes. Eggs were fertilized at 40 min after 1-MeAde treatment. (A) Ten minutes after application of $3 \mu\text{M}$ cytochalasin D (Sigma-Aldrich, St. Louis, MO), which is applied to the fertilized eggs at 90 min after fertilization. (B) Fertilized eggs were pulse-treated with the cytochalasin ($3 \mu\text{M}$) for 10 min after the third cleavage. Each blastomere lost its contact with the fertilization membrane, and transformed its flattened shape into a globular one. Scale bar: $100 \mu\text{m}$.

which the tips of the fixing processes were joined (Fig. 6D, G). Near the cleavage furrows (Fig. 9), the inner layer was occasionally seen detached from the fertilization envelope and was linked with a number of fixing processes (arrows).

Discussion

In the early cleavage stages before the establishment of some mechanism, such as desmosomes, to directly bind blastomeres, animal embryos devise various means to keep the blastomeres together and maintain their spatial positions relative to each other. In sea urchin eggs, a hyaline layer is formed by the exocytosis of cortical granules immediately after fertilization (Endo, 1961). Microvilli are embedded into the hyaline layer, thus fixing the surface of fertilized eggs to the hyaline layer. Each blastomere produced by cleavage is therefore held in place by the hyaline layer (Dan and Ono, 1952) by means of microvilli, thereby maintaining the three-dimensional spatial relationship among the blastomeres. In sea urchin embryos, even if the fertilization envelope is removed, normal morphogenesis continues as long as the hyaline layer remains (Showman and Foerder, 1979).

In this study, we found that in *Astropecten scoparius* embryos, which lack a hyaline layer, the relative positions among blastomeres are maintained by the fertilization envelope and the cytoplasmic threads (fixing processes) attached to it. As can be seen in Figure 6, fixing processes are altered microvilli. On fertilization, the tips of the microvilli buried in the vitelline coat are attached to the fertilization

envelope, and the microvilli rapidly lengthen with elevation of the fertilization envelope. Because fixing processes are located all over the cell surface, the balance of their tensions may keep the egg placed in the center of the fertilization envelope at the one-cell stage before the first cleavage. Hiramoto (1954, 1955) predicted the presence of a certain structure that connects the egg proper and the fertilization envelope in sea urchin zygotes, but its true nature is unknown.

After maturation division begins in *A. scoparius* oocytes, microfilament bundles within the microvilli lengthen, protruding some distance into the cytoplasm from the bases of the microvilli in unfertilized oocytes. Such lengthening following maturation division can be thought to be preparation for rapid elongation of microvilli postfertilization. Repeated bands were observed crossing the microfilament bundles (Fig. 6B, black arrowheads). The interval between the strips is some 60 nm. These bands may be formed of a kind of fascins, a family of actin-bundling proteins, although the band interval is wider than that reported in other types of cells (11 nm) (see Edwards and Bryan, 1995, for review).

After the first cleavage, the fixing processes near the cleavage furrow can be as long as $60 \mu\text{m}$. This rivals the length of the acrosomes of the sea cucumber *Dendrochirotyda thylene* (Tilney and Inoue, 1982)—a well-known example of the long actin fibers in gametes. In embryos after the second cleavage, fixing processes are short around the contact region between the blastomeres and the fertilization envelope (Fig. 6F). On the surface of the blastomeres in

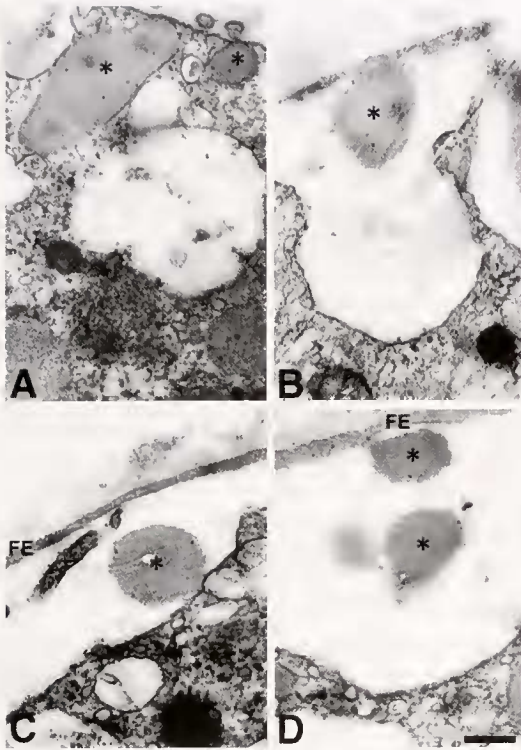


Figure 8. Immunoelectron microscopy of perivitelline-space particles using an antibody raised against sea urchin hyaline layer in chemically fixed specimens. (A) A maturing unfertilized oocyte. Cortical granules (asterisks), which were seen along the cortex, were decorated with gold particles. (B) Thirty seconds after insemination at 40 min after 1-MeAde treatment. Gold particles specifically decorated the cortical granule contents (asterisk) being discharged into the perivitelline space. (C) Five minutes after insemination. A perivitelline-space particle (asterisk) was decorated with gold particles. (D) Ten minutes after insemination. The fertilization envelope (FE) became decorated with gold particles after the perivitelline-space particles (asterisks) reached the envelope. Scale bar: 500 nm.

contact with the fertilization envelope, fixing processes are so short that they are difficult to detect by light microscopy (see Figs. 4D and 5A). Such short processes are thought to be the product of reduction of the long fixing processes seen before the first cleavage, although the mechanism of the reduction is not known.

Before the first cleavage, perivitelline-space particles reach the fertilization envelope and form its inner layer (Fig. 8D). As can be seen in Figures 6 and 8, this inner layer is derived from substances contained in the cortical granules, and the tips of the fixing processes are attached to the layer. Near the cleavage furrows, the inner layer, with fixing processes attached, could sometimes be seen detached from the fertilization envelope (Fig. 9), suggesting that the inner layer, derived from cortical granule contents, reinforces the attachment of the fixing processes to the fertilization envelope.

Treatment with acidified seawater breaks the attachments between the blastomeres and the fertilization envelope. The

effects of acidified seawater are not seen on embryos more than 6.5 h postfertilization, indicating that after that time, the fixing processes play a lesser role in holding the blastomeres in place. Intercellular binding devices such as desmosomes are probably fulfilling this role at this stage (*cf.* Dan-Sohkawa *et al.*, 1986). It is not known when the connections between fixing processes and the fertilization envelope are broken, but around the time of hatching the embryo is able to spin around inside the fertilization envelope, so the attachments have been severed by this time at the latest.

The spatial relationship between blastomeres is maintained by the hyaline layer and microvilli in sea urchin embryos, and in *A. scoparius* embryos, which lack a hyaline layer, by the fertilization envelope and microvilli. The morphology and location of the hyaline layer and the fertilization envelope are different, but each contains substances derived from cortical granules. It is of great interest that, as shown in the immuno-electron microscopic findings (Fig. 8), they contain some common elements. This way of using the fertilization envelope as a scaffold for morphogenesis, which has been seen in *A. scoparius* embryos, may be a

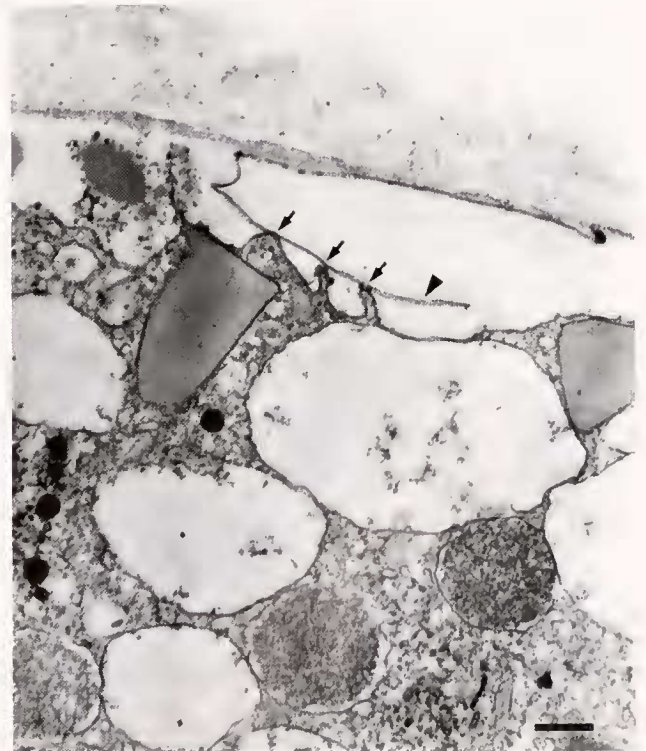


Figure 9. Detachment of fixing processes from the fertilization envelope during cleavages. Transmission electron micrograph of the specimen chemically fixed at the third cleavage. Near the cleavage furrow, the innermost layer of the fertilization envelope (arrow), to which fixing processes (arrowheads) were becoming attached, was ripped off from the fertilization envelope proper. The cleavage furrow was on the left side of the blastomere. Scale bar: 500 nm.

common mechanism for starfish embryos that lack a hyaline layer in early cleavage stages.

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